# Synergistic Activation of the Tumor Suppressor, HLJ1, by the Transcription Factors YY1 and Activator Protein 1

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## Abstract

**HLJ1 is a novel tumor and invasion suppressor that inhibits tumorigenesis and cancer metastasis. However, the mechanism of HLJ1 activation is currently unclear. Here, we identify an enhancer segment in the *HLJ1* gene at −2,125 to −1,039 bp upstream of the transcription start site. A 50-bp element between −1,492 and −1,443 bp is the minimal enhancer segment, which includes the activator protein 1 (AP-1) site (−1,457 to −1,451 bp), an essential regulatory domain that binds the transcriptional factors FosB, JunB, and JunD. Chromatin immunoprecipitation assays confirm that these AP-1 family members bind to a specific site in the HLJ1 enhancer segment *in vivo*. Overexpression of either YY1 at promoter or AP-1 at enhancer results in a 3-fold increase in the transcriptional activity of HLJ1. We propose a novel mechanism whereby expression of the tumor suppressor, HLJ1, is up-regulated via enhancer AP-1 binding to promoter YY1 and the coactivator, p300, through DNA bending and multiprotein complex formation. The combined expression of AP-1 and YY1 enhances HLJ1 expression by more than five times and inhibits *in vitro* cancer cell invasion. Elucidation of the regulatory mechanism of HLJ1 expression may facilitate the development of personalized therapy by inhibiting cancer cell proliferation, angiogenesis, and metastasis.** [Cancer Res 2007;67(10):4816–26]

## Introduction

Heat shock proteins (HSP), involved in the fundamental defense mechanism for maintaining cellular viability, are markedly induced during environmental or pathogenic stress (1, 2). Under normal conditions, HSPs perform essential functions, such as modulating activity by altering protein conformation, serving as molecular chaperones, promoting multiprotein complex assembly and disassembly, and ensuring proper protein folding (3–5). HSPs additionally function in immunologic processes, cell cycle regulation, transcriptional activation, signal transduction, and oncogenesis (2, 6–8).

HLJ1, also designated DNAJB4 in GenBank, has been cloned and classified as belonging to the HSP40 family (HSP40/DnaJ; ref. 9). HSP40 proteins occur ubiquitously in cells and display significant diversity in eukaryotic genomes (10, 11), with at least 44 genes in the human genome (12). However, the biological properties of HLJ1 are poorly understood at present.

HLJ1 is a novel tumor suppressor (13) that inhibits cancer cell cycle progression, proliferation, anchorage-independent growth, motility, invasion, and tumorigenesis. Moreover, HLJ1 expression is associated with reduced cancer recurrence and prolonged survival in non–small cell lung cancer patients. In a previous study, we identified the *HLJ1* gene promoter sequence (−232 to +176) and four YY1 transcription factor-binding sites within this region critical for promoter function (14). However, the activation and up-regulation mechanisms of HLJ1 require further investigation.

The transcription factor activator protein 1 (AP-1) plays an important role in the regulation of tumorigenesis-related genes (15–17). The issue of whether AP-1 has a regulatory role in HLJ1 remains to be established. Here, we show that the transcriptional activation and up-regulation mechanisms of the novel tumor suppressor, HLJ1, involve interactions between the transcription factors AP-1 and YY1 that bind to the enhancer and basal promoter regions of the gene, respectively.

## Materials and Methods

**Cell culture.** The human lung adenocarcinoma cell line, CL1-0 (18), and two human hepatoma cell lines, HepG2 and PLC, were maintained at 37°C in a humidified atmosphere of 5% $CO_2$. CL1-0 cells were cultured in RPMI 1640 (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc.) and 1% penicillin-streptomycin (Life Technologies, Inc.). HepG2 [American Type Culture Collection (ATCC) CRL-8024] and PLC (ATCC HB-8065) cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin.

**Cloning and sequencing of the enhancer region of the *HLJ1* gene.** A PCR-based method was used to clone the putative enhancer region of *HLJ1*. Specific primers were designed, based on the 5′-end of the known *HLJ1* promoter sequence identified in a previous study (14) and a bioinformatics search in GenBank. CL1-0 genomic DNA isolated using a QIAamp DNA blood mini kit (Qiagen) served as a PCR template. The sequences of the primer set employed in PCR are as follows: HLJP-F, 5′-CCGCTCGAGATTACGATTCTTATGTGTGTG-3′, introducing a *Xho*I site (underlined), and HLJPRE-R', 5′-CCCAAGCTTTTCGAATGCCTTGAAATTAAC-3′, containing a new *Hin*dIII site (underlined). The amplified 2,302-bp DNA fragment was digested with *Xho*I/*Hin*dIII and cloned into the promoterless pGL3-Basic vector (Promega) to generate pGL3-FRER'. The construct was verified by sequencing. Homology searches were done using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information.[8] Putative transcription factor binding elements in the *HLJ1*

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J.J.W. Chen and P.-C. Yang codirected the project and contributed equally.

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[8] http://www.ncbi.nlm.nih.gov

enhancer were analyzed with the MatInspector 2.2 (19) and SignalScan (20) programs,[9] using the TRANSFAC database (21).

**Construction of luciferase reporter gene constructs.** For luciferase assays, various lengths of the 5′-flanking region of *HLJ1* were generated by PCR using the pGL3-FRER′ clone as the template. Common reverse and different forward primers (specified in Table 1) were employed for the amplification of deletion fragments. *Xho*I and *Hin*dIII restriction sites were introduced in the forward and reverse primers, respectively, and employed for cloning the deletion fragments upstream of the luciferase reporter gene in the promoterless pGL3-basic vector. pGL3-Control, a positive control plasmid, was obtained from Promega. The putative enhancer (−2,125 to −1,039 bp) element and various deletion mutants (Fig. 2) generated by PCR were subcloned into the pGL3-promoter vector containing the luciferase gene under the control of the SV40 promoter. A similar cloning strategy was used to generate the minimal enhancer construct, pGL3-p-Emi. The pGL3-p-Emi construct served as a template for generating mutations in the Sp1 and AP-1 binding sites. All mutant constructs were prepared by PCR using the appropriate primers (Table 1). Constructs with different enhancer orientations and positions were generated by standard restriction enzyme digestion and cloning techniques. All PCR primers used for generating reporter gene constructs are listed in Table 1. Constructs were confirmed by restriction endonuclease digestion and DNA sequencing.

**Transfection and luciferase assays.** All transfections were done in triplicate in six-well plates. About $2 \times 10^5$ cells per well were seeded for 24 h before transfection. Plasmids were transfected into cells using the LipofectAMINE reagent according to the manufacturer's instructions (Invitrogen). The luciferase reporter constructs described above, along with the control plasmid, were cotransfected with a β-galactosidase construct, pSV-β-Gal (Promega) at a DNA ratio of 3:1. Cotransfection experiments involved a constant amount of *HLJ1* enhancer-reporter luciferase plasmid or pGL3-promoter vector DNA and AP-1 expression plasmids at different ratios plus 1 μg of internal control, pSV-β-Gal. The pcDNA3-FosB, pcDNA3-JunB, pcDNA3-JunD, and pcDNA3-Fra1 expression constructs were generated by inserting full-length cDNA into the pcDNA3 vector (Invitrogen). Cells were incubated in the transfection mixture for 4 h and harvested after 44 h in culture. An aliquot of cell lysates (10–25 μL) was used to assay luciferase activity using a luciferase assay kit (Tropix, Inc.). Another aliquot (10–25 μL) was used to measure β-galactosidase activity with the Galacto-Light chemiluminescent assay kit (Tropix, Inc.). Luminescence was measured using a Victor$^2$ 1420 Multilabel Counter (Perkin-Elmer). Transfection efficiency was normalized with β-galactosidase activity. Each experiment was repeated at least thrice.

**Electrophoretic mobility shift assay.** Double-stranded oligonucleotides were prepared by heating at 80°C for 20 min before slow cooling to room temperature. Oligonucleotides were labeled using [γ-$^{32}$P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase. Labeled probes were purified from unincorporated [γ-$^{32}$P]ATP using MicroSpin G-25 columns. Nuclear extracts (5 μg of protein) were incubated for 20 min at room temperature in binding buffer [4% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris-HCl (pH, 7.5), and 0.1 μg of poly(d[I-C])] containing γ-$^{32}$P-end-labeled, double-stranded oligonucleotide in a final volume of 10 μL. Samples were resolved by electrophoresis on 4% polyacrylamide gels at 110 V in 1× Tris/borate/EDTA buffer for 150 min at 4°C. Gels were dried and placed on a phosphoimage screen overnight. For competition assays, unlabeled oligonucleotides were added to binding reagents at 100-fold molar excess for 10 min before the addition of radiolabeled probe. For antibody supershift analysis, binding reactions were incubated with 2 μg of c-Jun (sc-1694X), JunB (sc-46X), JunD (sc-74X), c-Fos (sc-52X), Fos-B (sc-48X), Fra1 (sc-605X), and Fra2 (sc-604X) antibodies (Santa Cruz Biotechnology) for 30 min at 4°C before the addition of the probe. The following oligonucleotides were used: AP-1–WT: AAAGAATTGCTGAATCATCATTGC-T, and AP-1–Mut: AAAGAATTGC**TAACTAA**TCATTGCT. Mutations were introduced into the wild-type (WT) AP-1 binding sites (bold and underlined).

**Table 1.** Primer sequences used for the construction of HLJ1 enhancer fragments of different lengths

| Amplification primer | Sequence (5′ to 3′)* |
|---|---|
| Enhancer forward primers | |
| HLJP-F | CCGCTCGAGATTACGATTCTTATGTGTGTG |
| HLJP-F1 | CCGCTCGAGAATTTTGAAGAGTAGAA AATCGTA |
| HLJP-F2 | CCGCTCGAGGGATTACCTAAAATGATA TTATAGG |
| HLJP-F5 | CCGCTCGAGCATTTGTCCTGTTTAATT AGGAAA |
| HLJP-EF | CGACGCGTATTACGATTCTTATGTGTGTG |
| HLJP-EF1 | CGACGCGTAGAACAATTTCCGGTT |
| HLJP-EF2 | CGACGCGTTTGATATTATTTCTTGGTGA |
| HLJP-EF3 | CGACGCGTTTCTTATTTATCTCTCTAATAG |
| HLJP-EF21 | CGACGCGTCCTCTGTAACCTACAGGTAG |
| HLJP-EF22 | CGACGCGTATGGTGTTGTTAAAGTAGAGA |
| HLJP-EF23 | CGACGCGTAAAATGCACAAAGATGAACAT |
| HLJP-EF24 | CGACGCGTTGGCATATAGAGTAGGCGTT |
| HLJP-EF25 | CGACGCGTTTACCCTTTATTATATTCTAAACA |
| HLJP-EF26 | CGACGCGTAAGGTTTTCTAACATTTTATTTG |
| HLJP-Emi-F | CGACGCGTAAAATGCACAAAGATGA |
| HLJP-Emi-SPF1 | CGACGCGTAAAAGTACTAAAGATGA |
| Enhancer reverse primers | |
| HLJPRE-R′ | CCCAAGCTTTTCGAATGCCTTGAAATTAAC |
| HLJP-ER | CCGCTCGAGCCTATAATATCATTTTAGGTA |
| HLJP-ER1 | CCGCTCGAGCTATTAGAGAGATAAATAAG AAAAGTCA |
| HLJP-ER2 | CCGCTCGAGTCACCAAGAAATAATATCAA |
| HLJP-ER3 | CCGCTCGAGAACCGGAAATTGTTCT |
| HLJP-Emi-R | CCGCTCGAGAGCAATGATGATTCAG |
| HLJP-Emi-APR1 | CCGCTCGAGAGCAATGATTAGTTAGC |

*Restriction enzyme sites located within the PCR primers are underlined. *Xho*I site: CTCGAG; *Hin*dIII site: AAGCTT; *Mlu*I site: ACGCGT.

**Chromatin immunoprecipitation assays.** A chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology) was employed according to the manufacturer's instructions. CL1-0 ($1 \times 10^6$) cells were cross-linked in a 1% formaldehyde solution for 10 min at 37°C. Cells were lysed in 200 μL of SDS buffer and sonicated to generate 200–1,000 bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with ChIP buffer and incubated with the indicated antibodies at 4°C. Immune complexes were precipitated, washed, and eluted as recommended by the manufacturer. DNA-protein cross-links were reversed by heating at 65°C for 4 h. Next, DNA fragments were purified and dissolved in 30 μL of water. An aliquot of each sample (1 μL) was used as the template for PCR, along with HLJP-EF21 and HLJP-ER1 primers. This primer set encompasses the *HLJ1* enhancer segment from nucleotides −1,591 to −1,295, which includes the AP-1 binding site.

**Matrigel invasion assay.** The invasiveness of CL1-5 cells transfected with YY1 and AP-1 constructs was examined using the membrane invasion culture system, as described previously, with some modifications (14). Briefly, transwell membranes (8 μm pore size, 6.5 mm diameter; Corning Costar Corporation) were coated with Matrigel (2.5 mg/mL; BD Biosciences Discovery Labware). Cells were trypsinized, centrifuged, and resuspended at a density of $10^4$ cells/mL in RPMI containing 10% FBS and seeded onto the upper chambers of precoated transwells. Lower chambers of the transwells contained the same medium. After 18 h of incubation, membranes coated with Matrigel were swabbed with cotton, fixed with methanol, and stained with Giemsa stain (Sigma Chemical) before cell counting under phase-contrast microscopy.

[9] http://thr.cit.nih.gov/molbio/signal/ and http://www.genomatix.de/products/index.html.

**Coimmunoprecipitation and Western blot analysis.** Nuclear extract preparation and Western blot analysis methods have been described previously (22). Immunoprecipitation of endogenous YY1, JunB, JunD, and p300 was done as described below. CL1-0 cell nuclear extracts (1 mg) were diluted in lysis buffer. Lysates were precleared with control immunoglobulin G (IgG) and protein A/G PLUS agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. Precleared lysates were incubated with anti-YY1 monoclonal (Santa Cruz Biotechnology), anti-JunB polyclonal (Santa Cruz Biotechnology), anti-JunD polyclonal (Santa Cruz Biotechnology), or anti-p300 polyclonal antibody (Santa Cruz Biotechnology) overnight at 4°C, and immobilized on protein A/G agarose beads. Beads were washed five times with 1 mL of lysis buffer each. Total cell lysates were isolated from CL1-5 cells (14). HLJ1 and Fra1 were detected using anti-HLJ1 polyclonal (made in-house) and anti-Fra1 polyclonal antibodies (Santa Cruz Biotechnology). β-Tubulin was employed as the loading control. Endogenous proteins were resolved on SDS polyacrylamide gels and visualized with the imaging analyzer LAS3000 (Fujifilm, Tokyo, Japan).

**Statistical analysis.** All experiments were done in triplicate, and significant differences were analyzed by ANOVA (Excel, Microsoft). Data were considered statistically significant at $P < 0.05$. Where appropriate, results are presented as means ± SD.

## Results

**Identification of the *HLJ1* enhancer region.** In a previous study, we characterized the putative promoter region and investigated transcriptional regulation of the human *HLJ1* gene (14). Promoter activity of the plasmid encompassing the entire 1,214 bp upstream of the initiation codon (pGL3-F2RER') was approximately 27-fold higher than that of the pGL3-basic vector (Fig. 1). To gain further understanding of the events regulating *HLJ1* transcription, we extended the 5′-flanking region to 2.3 kb and analyzed transcriptional activity using the reporter gene assay. All constructs are presented in Fig. 1*A*. As shown in Fig. 1*B*, the plasmid containing the entire 2,301 bp upstream of the initiation codon (pGL3-FRER') displayed the highest luciferase activity in CL1-0 cells (approximately 500-fold higher), compared with the pGL3-basic vector (negative control). Sequential deletion of 603 bp from the 5′ end of the HLJ1 promoter region resulted in a 50% (pGL3-F1RER') and 80% (pGL3-F2RER') decrease in transcriptional activity, respectively, compared with the construct containing the entire 2,301-bp region (−2,125/+176). The promoter activity of pGL3-F2RER' was similar to that of the basal promoter construct (pGL3-F5RER').

To determine whether the transcriptional regulatory elements in *HLJ1* contain cell type–specific features, all constructs were transfected into two human hepatoma cell lines, HepG2 (Fig. 1*C*) and PLC (Fig. 1*D*). Because the *HLJ1* gene was isolated from the human liver cDNA library (9), its transcriptional regulation was functional in the two hepatoma cell lines. As expected, all *HLJ1* promoter constructs were functional in these two cell lines. Moreover, pGL3-FRER' and pGL3-F1RER' constructs displayed significantly higher transcriptional activity than the basal promoter construct (pGL3-F5RER').

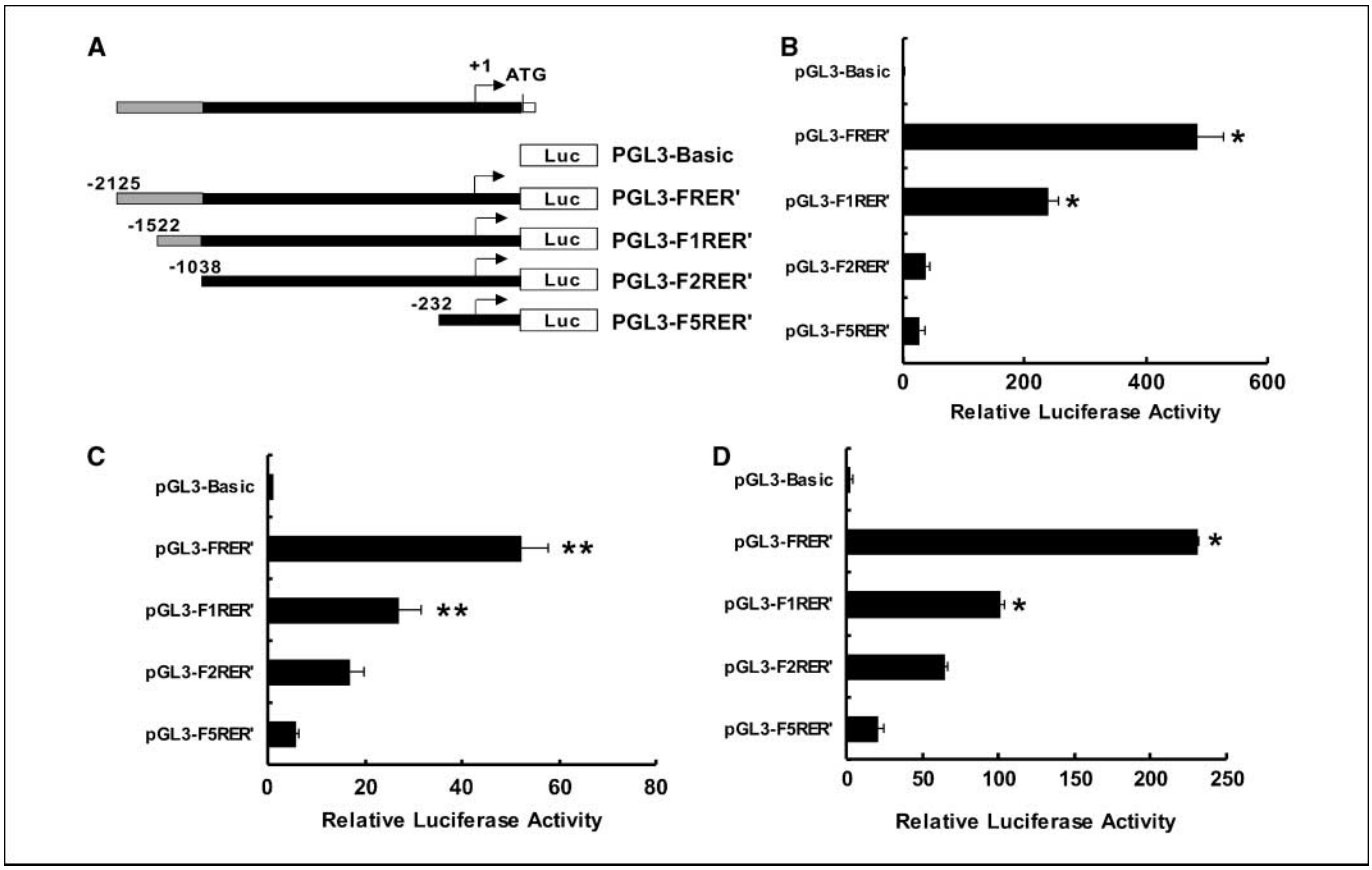


**Figure 1.** Human *HLJ1* promoter activity in different cell types. *A,* 5′ deletion constructs of the *HLJ1* promoter. Relative luciferase activities of pGL3-FRER' and pGL3-F1RER' (containing the potential enhancer element); pGL3-F2RER' and pGL3-F5RER' (containing full-length and basal promoter, respectively) were determined in several cell types: *B,* CL1-0 cells; *C,* HepG2 cells; *D,* PLC cells. Relative activities shown as bars on the right correspond to the constructs illustrated on the left. Results were correlated with luciferase activity from cotransfected pSV-β-Gal and expressed as relative luciferase activity. *Columns,* means from three separate experiments; *bars,* SD. *, $\alpha = 0.05$, $P < 0.001$; **, $\alpha = 0.05$, $P < 0.05$, compared with pGL3-F5RER'.

The above results clearly suggest that the region between −2,125 and −1,039 functions as an enhancer element.

To confirm the presence of an enhancer region between −2,125 and −1,039 bp, various lengths of this region were subcloned into the enhancerless pGL3-SV40-promoter vector to generate pGL3-p-EFR, pGL3-p-EF1R, pGL3-p-EF2R, and pGL3-p-EF3R (Fig. 2*A*). These 5′-end deletion constructs were used for transient transfection of CL1-0 cells, and transcriptional activation was determined by the measurement of luciferase activity. The vector containing the complete sequence (pGL3-p-EFR) displayed approximately 12-fold higher luciferase expression, compared with the empty pGL3-promoter vector, as depicted in Fig. 2*A*.

Further stepwise removal of sequences spanning −2,125 and −1,295 bp led to a marked decrease in luciferase expression. To identify the minimal *HLJ1* enhancer region, six other deletion fragments were cloned into the pGL3-SV40-promoter vector. Interestingly, the pGL3-p-EFR1 construct with a 3′-end deletion exhibited the highest enhancer activity (about 51-fold), relative to the empty pGL3-promoter vector ($\alpha = 0.05$, $P = 0.002$). However, further 3′-end deletion (pGL3-p-EFR2, pGL3-p-EFR3) resulted in a dramatic decrease in enhancer activity. These results signify the presence of a silencing element within 256 nucleotides at the 3′-end (−1,295 to −1,039 bp).

Bidirectional deletion of the putative enhancer region revealed that a 346-nucleotide fragment (pGL3-p-EF2R1) produces transcriptional activity of luciferase 2-fold ($\alpha = 0.05$, $P = 0.032$) and 19-fold ($\alpha = 0.05$, $P = 0.003$), compared with the entire 1,087-bp enhancer region (pGL3-p-EFR) and empty pGL3-promoter vector, respectively. Based on the results, we propose that this 346-nucleotide region (EF2R1) is the minimal domain of the *HLJ1* enhancer.

To ascertain that these fragments fulfill the requirements of an enhancer, EFR1 and EF2R1 were inserted into the pGL3-promoter vector in the sense or antisense orientation. Notably, luciferase activity was stimulated 20-fold by pGL3-p-EIFR1 and 10-fold by pGL3-p-EIF2R1 (Fig. 2*A*), thus providing additional evidence that these fragments function as effective enhancer segments.

**Interactions between the 5′-enhancer and *HLJ1* basal promoter.** To further clarify the positional effect between the enhancer and basal promoter regions, the sequence between −1,038 and −232 was deleted to generate the pGL3-EFR-F5RER' plasmid. As shown in Fig. 2*B*, this recombinant enhancer-promoter construct efficiently stimulated luciferase expression (about 18-fold), compared with the *HLJ1* basal promoter construct (pGL3-F5RER'). However, the recombinant construct accounted for only 36% of the luciferase activity, compared with the 2,301-bp full-length HLJ1 enhancer-promoter construct (pGL3-FRER'). Without the basal promoter, the construct containing the enhancer alone did not induce any available transcriptional regulation (pGL3-EFR).

A recombinant construct containing the enhancer sequence between −2,125 and −1,295 bp (pGL3-EFR1-F5RER') displayed similar luciferase activity to pGL3-EFR-F5RER'. This finding indicates that the silencing element loses its regulatory function under the positional effect. Furthermore, the minimal domain of the *HLJ1* enhancer (EF2R1) stimulated luciferase reporter gene expression about 8.5-fold, compared with pGL3-F5RER' ($\alpha = 0.05$, $P = 0.002$). In view of these results, we conclude that the *HLJ1* enhancer functions in a position-dependent manner.

**The *HLJ1* minimum enhancer is located in the 5′-flanking region between −1,492 and −1,443 bp.** To identify the minimal functional enhancer element, a series of 5′-end deletion fragments overlapping the EF2R1 (−1,641 to −1,295) region were generated by PCR. Fragments were subcloned into the pGL3-promoter vector and transfected into CL1-0 cells to measure luciferase reporter activity (Fig. 2*C*). Four constructs (pGL3-p-EF2R1, pGL3-p-EF21R1, pGL3-p-EF22R1, and pGL3-p-EF23R1) presented strong enhancer activity, whereas three others (pGL3-p-EF24R1, pGL3-p-EF25R1, and pGL3-p-EF26R1) displayed low enhancer activity. We observed a dramatic loss (85%) in enhancer activity when the region between −1,492 and −1,443 bp was deleted (EF24R1). Furthermore, when this region was subcloned (pGL3-p-Emi) and enhancer activity assay was done, an approximate 23-fold increase in reporter gene expression was evident, compared with the empty pGL3-promoter vector ($\alpha = 0.05$, $P = 0.007$). The results collectively imply that the 50-bp fragment between −1,492 and −1,443 bp in the 5′-flanking region of *HLJ1* is the minimum enhancer element.

**The AP-1 motif is required for positive enhancer activity of *HLJ1*.** The nucleotide sequence of the full-length *HLJ1* enhancer element (−2,125 to −1,039) reported here (Fig. 3) has been deposited in the GenBank database with accession number DQ375392. Several potential transcriptional elements were identified within the EF2R1 element with the MatInspector v2.2 program (core similarity 0.8, matrix similarity 0.9) using TRANSFAC matrices (ref. 19; Fig. 3), including potential binding sites for GR, Pit-1, NF-E2, HiNF-A, Sp1, AP-1, and GATA-1.

Only two potential transcriptional elements exist within the minimum *HLJ1* enhancer element region, specifically, a non-canonical Sp1 binding site at the 5′-end and an AP-1 site at the 3′-end. To determine whether these elements are required for *HLJ1* enhancer function, three mutants with nucleotide substitutions in the AP-1 and/or Sp1 binding sites were generated using the minimum enhancer element as a template (Fig. 4*A*). The mutant constructs were transfected into CL1-0 cells to evaluate enhancer activity. As depicted in Fig. 4*B*, nucleotide substitution in the AP-1 site (pGL3-p-Emi-F/APR1) resulted in a significant decrease in enhancer activity, compared with the basal level of the pGL3-promoter vector.

However, substitutions in the noncanonical Sp1 binding site (pGL3-p-Emi-SPF/R) did not significantly affect *HLJ1* enhancer activity. Mutations in both the AP-1 and Sp1 binding sites (pGL3-p-Emi-SPF/APR1) resulted in a decrease in enhancer activity to the level of the AP-1 mutant construct. The results support the theory that the AP-1 binding site is involved in the up-regulation of *HLJ1* enhancer activity (Fig. 4*B*).

**A specific AP-1 protein complex increases *HLJ1* enhancer activity.** To determine whether the AP-1 protein binds to the putative site in *HLJ1*, an electrophoretic mobility shift assay was done. Wild-type or mutant oligonucleotides encompassing the AP-1 site (−1,467 to −1,443 bp) were radiolabeled, incubated with nuclear extracts from CL1-0 cells, and analyzed by nondenaturing PAGE (Fig. 4*C*). DNA-protein binding complexes were obtained with wild-type oligonucleotides (lane 3), but not mutated oligonucleotides (lane 1). In a competition assay, excess unlabeled wild-type oligonucleotides competed for complex formation (lanes 4 and 5), but not a nonspecific DNA probe containing an YY1 binding site (data not shown). In addition, upon substitution of the potential AP-1 binding site, the mutant AP-1 oligonucleotide lost its competitive ability (data not shown).

We next examined the composition of the AP-1 protein complex in CL1-0 cells using antibodies specific for individual Jun (c-Jun, JunB, and JunD) or Fos (c-Fos, FosB, Fra1, and Fra2) proteins. In subsequent supershift analyses, antibodies against FosB, JunB, and JunD led to the disappearance of the AP-1–specific band (lanes 7,

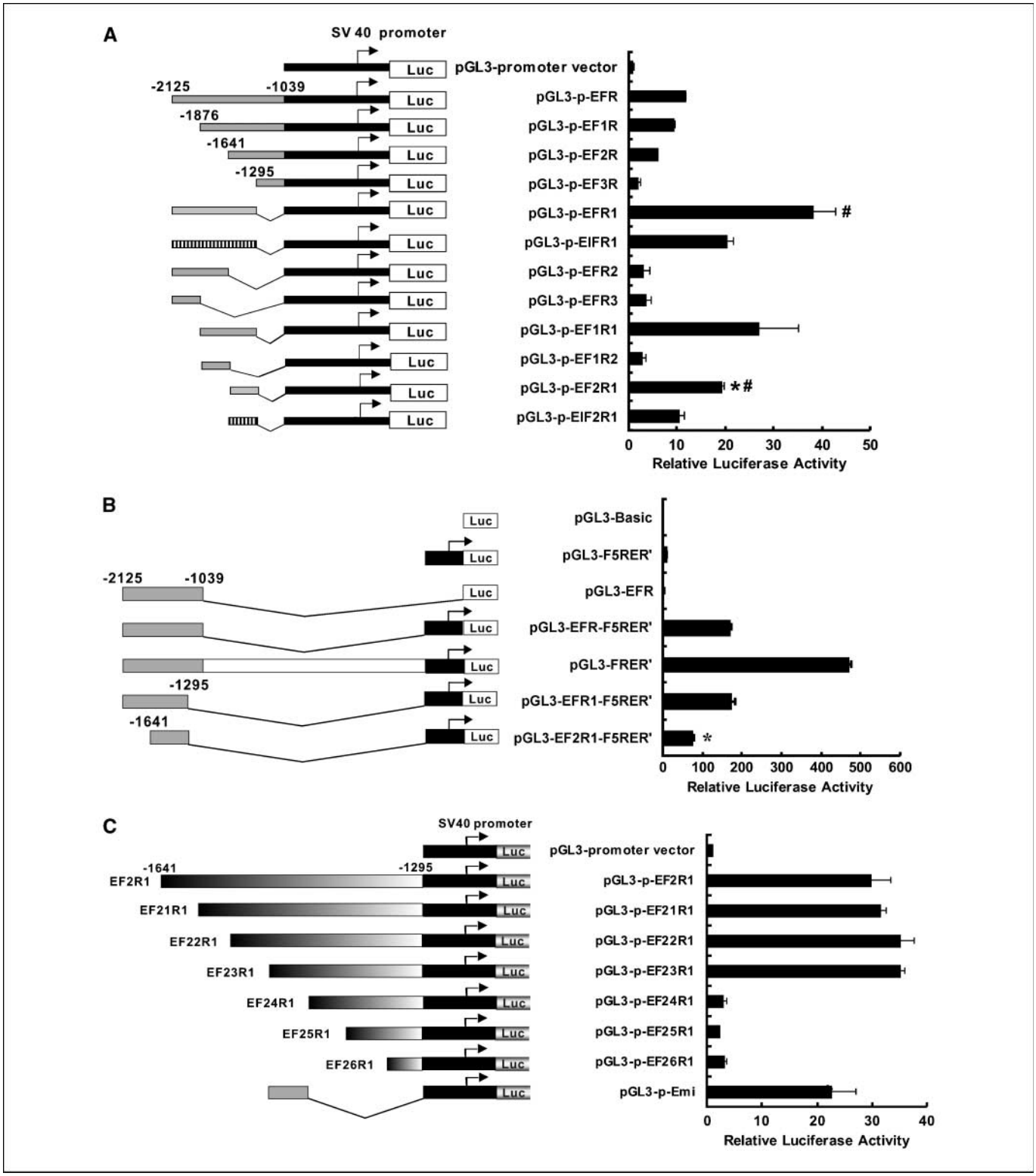


**Figure 2.** Functional deletion mapping of human *HLJ1* enhancer minimal domain. *A, left, HLJ1* promoter deletion mutants cloned upstream of the luciferase gene in the pGL3-promoter vector. EFR1 and EF2R1 fragments were subcloned into the pGL3-promoter vector in the reverse orientation to generate pGL3-p-EIFR1 and pGL3-p-EIF2R1, respectively. The numbers on the left of each enhancer deletion construct refer to the start position of the promoter fragments. Each construct was transiently cotransfected with the pSV-β-Gal vector into CL1-0 cells. *Right,* graph of luciferase activities. Activity values were normalized to β-galactosidase activity and presented as fold increase relative to the pGL3-promoter vector. *, $\alpha = 0.05$, $P < 0.05$ compared with pGL3-p-EFR. #, $\alpha = 0.05$, $P < 0.005$, compared with pGL3-promoter vector control. *B,* promoter activity assay of *HLJ1* enhancer-basal promoter recombinant constructs. CL1-0 cells were transiently transfected with each *HLJ1* enhancer-basal promoter recombinant construct. The luciferase activity obtained for each construct was normalized to that of pGL3-Basic. *, $\alpha = 0.05$, $P < 0.005$, compared with pGL3-F5RER'. *C,* fine 5′ deletions of −1,641/−1,295 bp HLJ1-luciferase construct (pGL3-p-EF2R1) show that enhancer activity mainly resides in a 50-bp element from −1,492 to −1,443 bp (pGL3-p-Emi). Each construct was transiently cotransfected with the pSV-β-Gal vector into CL1-0 cells. *Columns,* mean relative luciferase activities from three separate experiments; *bars,* SD. *, $\alpha = 0.05$, $P < 0.01$, compared with the pGL3-promoter vector control.

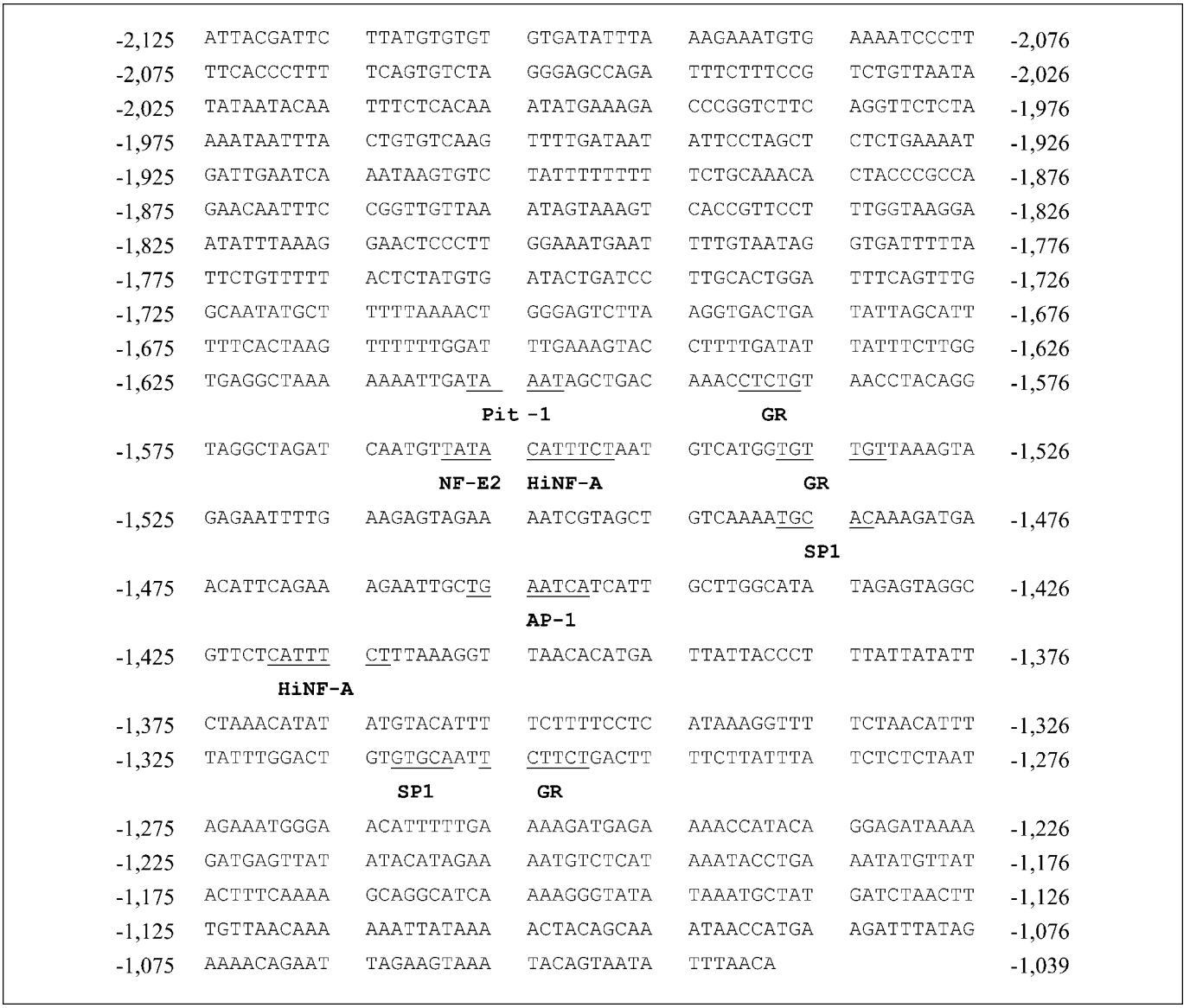


**Figure 3.** Nucleotide sequences of the putative enhancer region of the human *HLJ1* gene. A total of 1,087 bp of *HLJ1* 5′-flanking region was cloned and sequenced. The sequence is numbered, relative to the transcription start site. *Underlined,* putative transcription binding sites located within the minimum enhancer domain (−1,641 to −1,295); the binding transcription factors are specified below.

11, and 12). However, in the presence of the unrelated control goat anti-rabbit IgG and YY1 antibodies, the retarded band was not shifted.

To further distinguish the AP-1 family members that bind to the AP-1 site in the *HLJ1* enhancer *in vivo*, ChIP assays using specific antibodies were done. FosB, JunB, and JunD are the major AP-1 members that interact with the *HLJ1* enhancer (Fig. 4*D*). An anti-acetylhistone H3 antibody was used as a positive control, whereas IgG and anti-YY1 antibody were employed as negative controls. Our results suggest that this region is important for the regulation of *HLJ1* enhancer activity.

**Overexpression of JunB and JunD, but not Fra1, enhances HLJ1 levels.** To identify the specific transcription factors that regulate *HLJ1* enhancer activity, pcDNA3-FosB, pcDNA3-JunB, or pcDNA3-JunD and *HLJ1* enhancer constructs were cotransfected into CL1-0 cells and subjected to the enhancer activity assay. As shown in Fig. 5*A*, *HLJ1* minimum enhancer (pGL3-p-Emi) activity was positively correlated with cotransfected JunB or JunD in a concentration-dependent manner. However, FosB alone could not transactivate *HLJ1* enhancer activity. Moreover, the AP-1 mutant construct (pGL3-p-Emi-APR1) had no effect on *HLJ1* minimum enhancer activity, even upon JunB or JunD cotransfection, similar to the pGL3-promoter control.

We further investigated whether the activity of full-length *HLJ1* enhancer is regulated by the transcription factors, FosB, JunB, and JunD, under real physiologic conditions. The *HLJ1* promoter with the enhancer region (pGL3-FRER') was cotransfected with pcDNA3-FosB, pcDNA3-JunB, or pcDNA3-JunD into CL1-0 cells, respectively. Because Fra1 could not bind to the putative AP-1 site in the *HLJ1* enhancer in electrophoretic mobility shift assay (EMSA) analyses, the pcDNA3-Fra1 construct was employed as a negative control. As shown in Fig. 5*B*, JunB and JunD stimulated the transcriptional activity of the *HLJ1* promoter with the enhancer in a dose-dependent manner. The pcDNA3-Fra1 construct had no

effect on transcriptional activity. Moreover, FosB induced low transcriptional activity in a dose-dependent manner.

**Overexpression of JunB, JunD or YY1 stimulates HLJ1 expression and suppresses cancer cell invasion.** To further establish the role of AP-1 in *HLJ1* gene regulation, pcDNA3-AP-1 constructs were transiently transfected into highly invasive CL1-5 cells. As shown in Fig. 6*A*, JunB, JunD, or YY1 alone increased HLJ1 expression. In addition, various combinations of JunB, JunD, and YY1 enhanced HLJ1 protein expression. Combined expression of AP-1 and YY1 increased HLJ1 expression about 5.3-fold, compared with a 3-fold increase in HLJ1 expression in cells transfected with either YY1 or AP-1 alone.

To determine whether AP-1 affects cancer cell invasion, an *in vitro* invasion assay was done. In general, the invasive capabilities of transfectants with higher HLJ1 expression levels, without respect to JunB, JunD, YY1 alone, or various combinations of AP-1 and YY1-transfected cells, were significantly less than that of the mock transfectant ($\alpha = 0.05$, $P < 0.001$; Fig. 6*B*). Notably, cotransfection of JunB, JunD, and YY1 resulted in further suppression of invasive capability ($\alpha = 0.05$, $P = 0.000014$). However, CL1-5 cells transfected with pcDNA3-Fra1 had no effects on invasion, compared with CL1-5 or the mock transfectant.

**JunB and JunD are directly associated with YY1 on the *HLJ1* promoter.** Several reports show that YY1 cooperates with AP-1 in

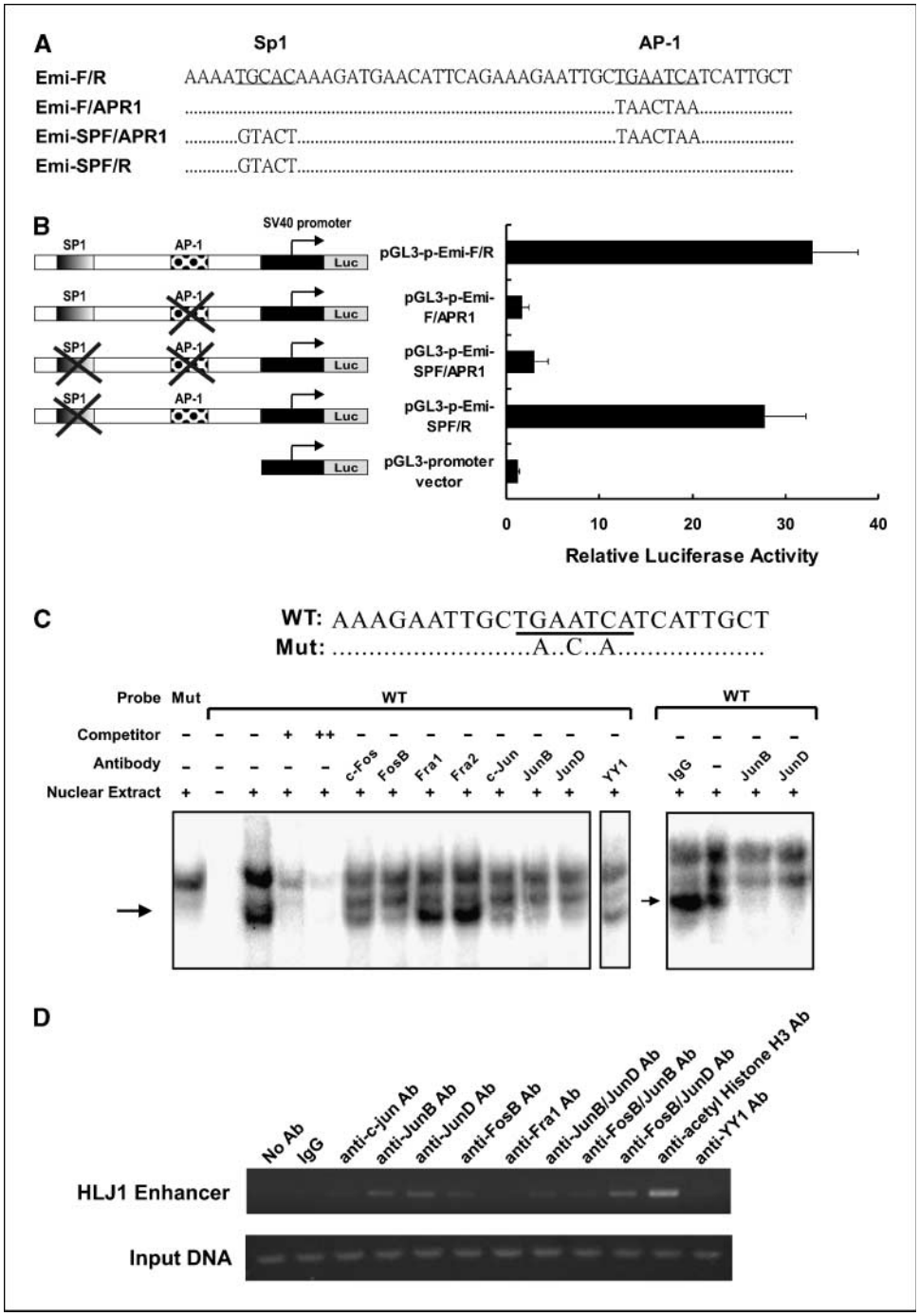


**Figure 4.** The AP-1 site is critical for *HLJ1* minimum enhancer activity. For mutation analysis of *HLJ1* minimum enhancer activity: *A,* the wild-type enhancer fragment and its mutant derivatives in which either the motif was substituted or a combination of motif mutations was fused to the pGL3-promoter vector. *B,* WT and mutant constructs were transfected into CL1-0 cells. Relative activities are depicted as bars on the right, corresponding to the constructs illustrated on the left. Results were correlated with luciferase activity from cotransfected pSV-β-Gal. *Columns,* relative luciferase activity means from three separate experiments; *bars,* SD. For EMSA analysis of the sequence encompassing the putative AP-1 site at −1,457 to −1,451 bp of the *HLJ1* gene: *C,* sequences of the WT or Mut (mutant) AP-1 oligonucleotide probes used in EMSA. Radiolabeled duplex probes were incubated with 5 μg CL1-0 cell nuclear extracts. *Arrow,* AP-1–immunoreactive complex. An AP-1–specific complex was formed with the wild-type probe, but not the mutant probe. Unlabeled WT oligonucleotide was used as a specific competitor. Formation of the AP-1 complex was completely eliminated by the addition of the FosB, JunB, and JunD antibodies. Competitor: +, 100 × WT; ++, 300 × WT oligonucleotides. Goat antirabbit IgG and anti-YY1 antibodies were used as the unrelated controls. *D,* ChIP analysis for *in vivo* binding of AP-1 in the *HLJ1* enhancer. Sheared chromatin fragments were immunoprecipitated with the indicated antibodies, and the *HLJ1* enhancer region was amplified by PCR. *No Ab,* no antibody; *IgG,* rabbit normal IgG; *input DNA,* PCR product of glyceraldehyde-3-phosphate dehydrogenase; *anti-acetyl Histone H3 Ab,* positive control.

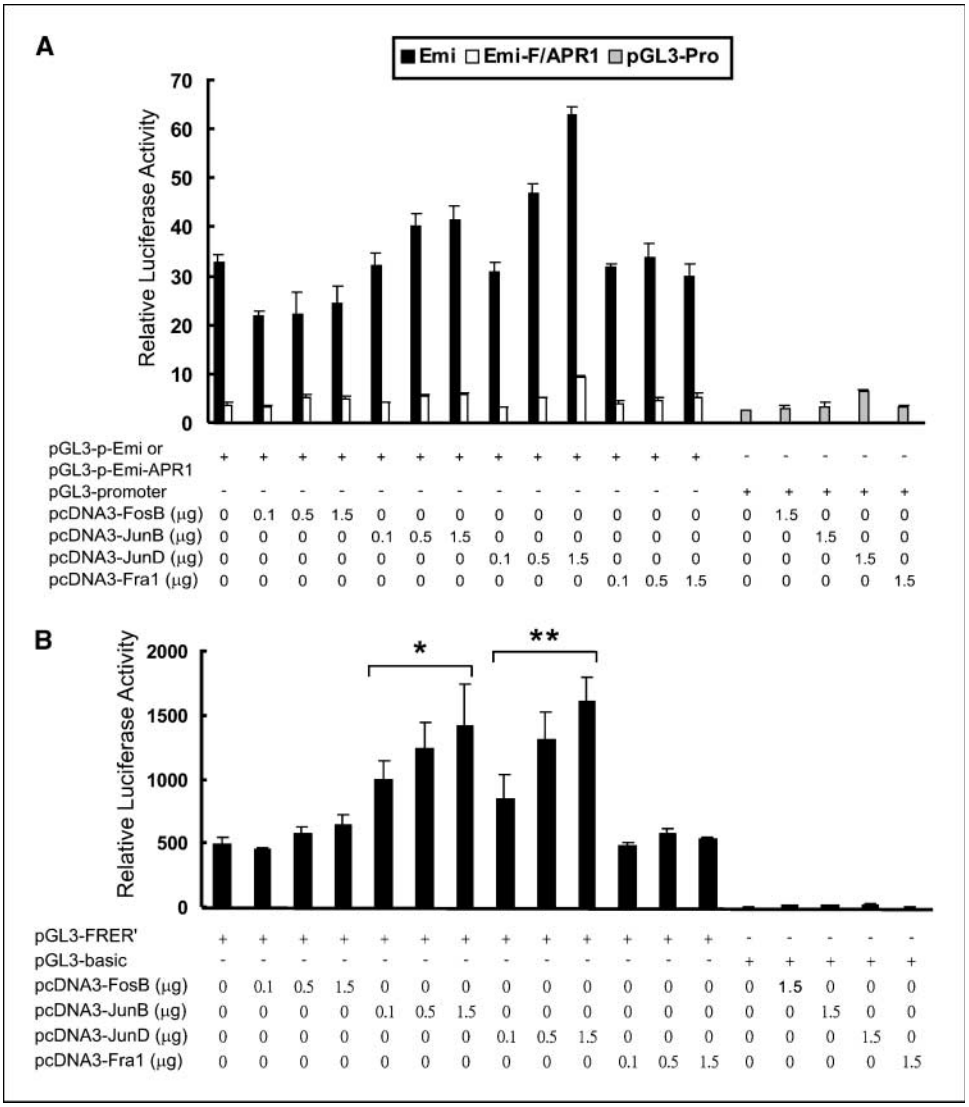


**Figure 5.** Overexpression of JunB and JunD stimulates *HLJ1* enhancer activity. *A,* CL1-0 cells were transiently cotransfected with wild-type (pGL3-p-Emi) or AP-1 mutant (pGL3-p-Emi-APR1) *HLJ1* minimum enhancer constructs and different concentrations of pcDNA3-FosB, pcDNA3-JunB, pcDNA3-JunD, or pcDNA3-Fra1 expression plasmids, using the LipofectAMINE method. *B,* the *HLJ1* full-length enhancer-promoter construct (pGL3-FRER') was cotransfected with various concentrations of pcDNA3-FosB, pcDNA3-JunB, pcDNA3-JunD, or pcDNA3-Fra1 into CL1-0 cells, and the promoter activity assay was done. *Columns,* means from three independent experiments; *bars,* SD. JunB and JunD increased luciferase activity in a dose-dependent manner. pGL3-promoter and pGL3-basic vectors were used as the negative controls. *, $\alpha = 0.05$, $P < 0.01$; **, $\alpha = 0.05$, $P < 0.05$ compared with pGL3-FRER'.

regulating gene expression (23, 24). To establish whether AP-1 interacts directly with YY1 on the *HLJ1* gene promoter, we selected JunB and JunD, which evidently stimulate transcriptional activity, for coimmunoprecipitation studies with CL1-0 cell nuclear extracts. JunB and JunD endogenously associated with YY1 in our experiments (Fig. 6*C*).

Furthermore, we propose that p300, a transcriptional coactivator that interacts with a variety of transcription factors, including AP-1 and YY1 (25), is a coactivator of AP-1 and YY1 in *HLJ1* gene expression. To confirm this hypothesis, interactions between endogenous p300 and AP-1 or YY1 were analyzed by coimmunoprecipitation experiments. Western blot analysis with a p300-specific antibody disclosed that immunoprecipitates obtained with AP-1 (JunB and JunD)-specific antibodies contained p300 protein. In contrast, those obtained with control IgG (preimmune serum) did not contain p300 protein (Fig. 6*C*).

## Discussion

HLJ1 is a novel tumor and invasion suppressor that inhibits tumor angiogenesis and cell cycle progression. The gene is used as an independent predictor of metastasis and survival in cancer patients (13, 14, 26). However, the mechanisms of HLJ1 activation and up-regulation are currently unclear. In a previous report, we showed that the *HLJ1* promoter contains four YY1-binding sites that positively regulate *HLJ1* expression (14). In this study, we further identify a novel enhancer segment in *HLJ1* at −2,125 to −1,039 bp upstream of the transcription start site. The 50-bp element at the 5′-flanking region (−1,492 to −1,443 bp) is the minimal enhancer segment, whereas the AP-1 site (−1,457 to −1,451) is an essential regulatory domain. The AP-1 motif is essential for the positive enhancer activity of *HLJ1*.

AP-1 is a nuclear transcription complex composed of dimers encoded by the *fos* and *jun* families of proto-oncogenes, which modulates transcription by binding to specific recognition motifs in the regulatory regions of target genes (27). The AP-1 complex modulates the transcriptional activation of a variety of genes through specific binding to the DNA sequence, "TGACTCA", designated an "AP-1 site". AP-1 plays important roles in the up-regulation of tumorigenesis-related genes (15–17). However, recent studies show that specific AP-1 proteins, such as JunB and c-Fos, have tumor suppressor activity, depending on the antagonistic activities of different Jun proteins, tumor type, stage, and genetic backgrounds (28). For example, elevated JunB expression in 3T3

cells is associated with the inhibition of Ras- and Src-mediated transformation and tumor growth *in vivo* (29). Among the Jun proteins, c-Jun positively regulates cell proliferation through the repression of tumor suppressor gene expression and function and induction of cyclin D1 transcription. These activities are antagonized by JunB (30). In addition, the tumor suppressor action of JDP2 is partially explained by the generation of inhibitory AP-1 complexes via an increase in JunB, JunD, and Fra-2 expression and decrease in c-Jun expression (31). A recent report showed that AP-1 heterodimers, such as c-Jun/Fra-1 or JunD/Fra-1, cooperate with the Ras oncogene in transcriptional activation of the tumor suppressor gene p14/p19$^{ARF}$ promoter upon oncogenic signaling in human cervical carcinoma cells (32). Our results confirm that FosB, JunB, and JunD bind to the putative AP-1 site in the *HLJ1* enhancer. HLJ1 expression is positively correlated with JunB and JunD expression in a dose-dependent manner. However, the composition of the AP-1 complex that activates *HLJ1* enhancer *in vivo* remains to be determined. Here, we initially disclose that homo- or heterodimers of FosB, JunB, and JunD bind to and activate the *HLJ1* enhancer *in vivo* (Fig. 4*D*). Nevertheless, to our knowledge, there are no obvious rules to clearly establish the AP-1 dimer types that activate specific tumor suppressors in different cells and tissues.

The transcription factor, YY1, up-regulates the tumor suppressor, HLJ1, by directly binding to the promoter region, thus inhibiting cancer cell invasion (14). YY1 is a complex protein that plays pivotal roles in development, differentiation, cellular proliferation, and apoptosis. Because the expression and function of YY1 are intimately associated with cell cycle progression, its physiologic significance has recently been applied to models of cancer biology. YY1 expression is additionally associated with tumorigenesis (33) and tumor recurrence in prostate cancer (34).

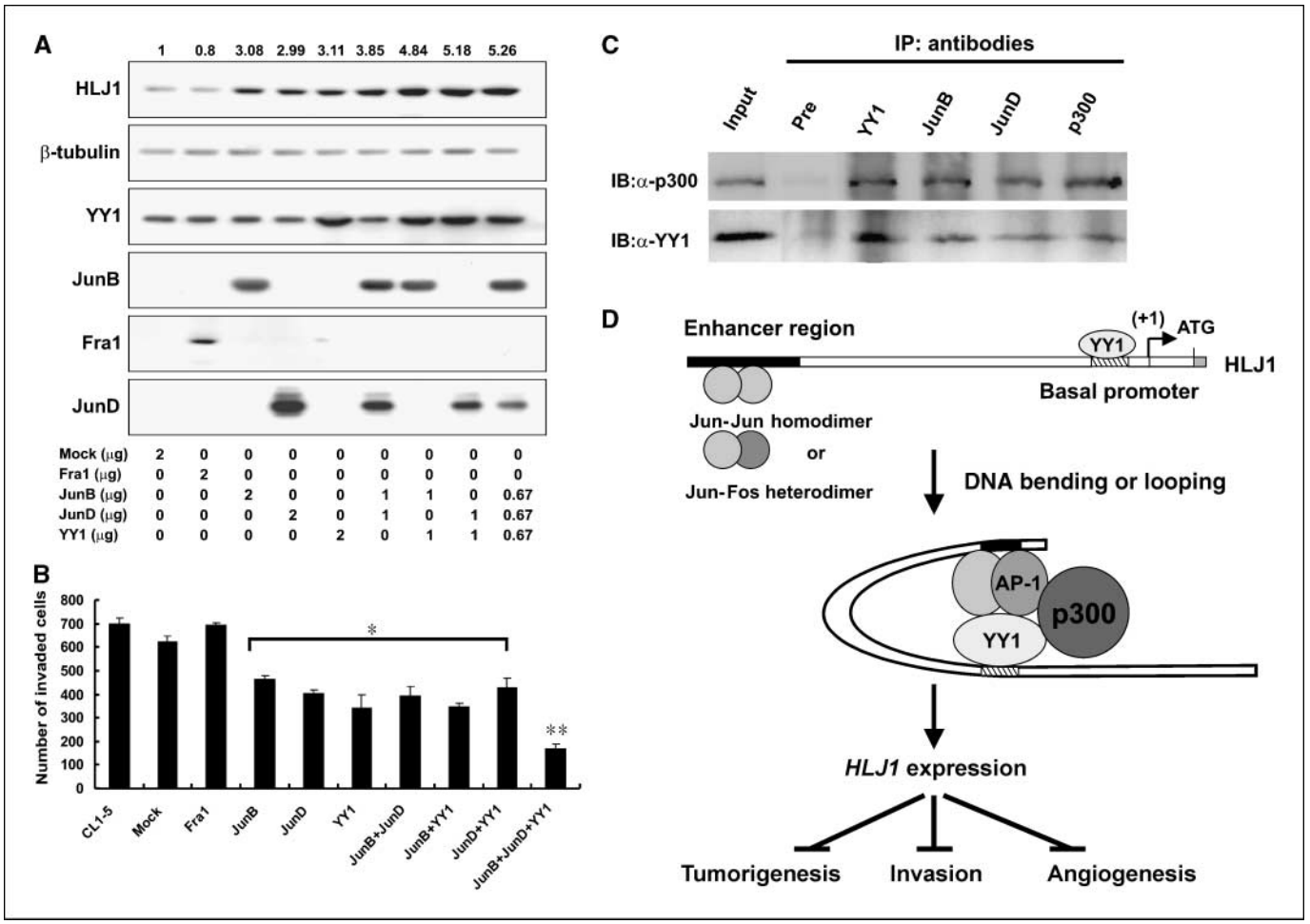


**Figure 6.** AP-1 and YY1 positively regulate HLJ1 expression and reduce the invasive capability of cancer cells. *A,* Western blotting analysis of HLJ1 expression was done in mock-transfected CL1-5, Fra1-, JunB-, JunD- or YY1-transfected cells, and various combinations of AP-1 and YY1-cotransfected cells. Expression of HLJ1 protein is indicated on the top in the first row. HLJ1 expression was higher in JunB-, JunD-, and YY1-transfected cells than in mock- and Fra1-transfected cells. *B,* overexpression of HLJ1 led to suppression of the *in vitro* invasion capability of CL1-5 cells. JunB-, JunD-, or YY1-transfected cells and cells cotransfected with various combinations of AP-1 and YY1 displaying higher HLJ1 protein levels significantly suppressed the invasion capability of cancer cells. *, $\alpha = 0.05$, $P < 0.001$; **, $\alpha = 0.05$, $P = 0.000014$, compared with mock-transfected cells. *Columns,* means of data from three independent experiments; *bars,* SD. *C,* coimmunoprecipitation of endogenous p300 and YY1 with AP-1. Nuclear extracts of CL1-0 cells were subjected to immunoprecipitation (*IP*), using antibodies specific for YY1 (*lane 3*), JunB (*lane 4*), JunD (*lane 5*), or p300 (*lane 6*). Precipitation done with a preimmune serum was employed as the negative control (*lane 2*). Immunoprecipitates and 0.5% lysates (*lane 1, input*) were fractionated by SDS-PAGE and analyzed by immunoblotting using the anti-p300 (*top*) or anti-YY1 (*bottom*) antibodies. *D,* a hypothetical model for the transcriptional regulation of *HLJ1*. We propose that AP-1 homodimers or heterodimers located in the enhancer region activate *HLJ1* gene transcription by interacting with YY1 at the promoter region. This interaction may be stimulated by the transcriptional cofactor, p300, that forms a multiprotein complex with AP-1 and YY1 through DNA bending or looping mechanisms.

However, the mechanisms responsible for the opposing oncogenic and tumor-suppressive properties of YY1 remain to be clarified.

In this study, we show that combined interactions of the enhancer-bound AP-1 and promoter-bound YY1, as well as p300 multiprotein complex formation, constitute a novel mechanism for up-regulation of the tumor suppressor, HLJ1. The combined expression of AP-1 and YY1 enhanced HLJ1 expression by 5.3-fold, compared with a 3-fold increase in HLJ1 expression by either YY1 at the promoter or AP-1 at the enhancer region. The results are consistent with earlier reports that these two transcription factors interact to regulate gene expression (23, 24).

The recombinant construct, pGL3-EFR-F5RER', displayed only 36% expression, compared with that containing the full-length *HLJ1* promoter region (pGL3-FRER'; Fig. 2*B*). These results indicate that the central region between −1,038 and −232 bp of the *HLJ1* promoter is necessary for intact promoter activity. Querfurth et al. (35) proposed a similar interaction model for amyloid precursor protein transcription regulation with DNA looping. In their model, DNA looping and synergistic enhancement of transcription occurs when the near-upstream and proximal domains interact. This model may explain why cotransfection of JunB, JunD, and YY1 induces a significant increase in HLJ1 expression. In addition, previous reports suggested that in the presence of an enhancer, genes may become supercoiled or looped for active transcription. DNA supercoiling may significantly increase communication between the enhancer and promoter regions over a long distance. Transcription factor interactions between a distal enhancer and proximal promoter may be favored in supercoiled DNA owing to topological constraints (36, 37). Thus, the location of the *HLJ1* enhancer at the 5′-end far upstream of the promoter may favor protein-protein interactions.

Figure 6*D* represents a summary of activation and synergistic up-regulation of HLJ1 by AP-1 binding at the enhancer and YY1 at the promoter region, multiprotein complex formation with AP-1, YY1, and transcriptional cofactor p300, DNA bending and spatial relationships between transcription activators and components of the basal transcription apparatus. Identification of the control mechanisms of a tumor suppressor protein may advance our understanding of cancer and facilitate the development of novel target therapy for cancer cell proliferation, angiogenesis, and metastasis. For instance, the majority of clinical studies suggest that lung cancers with p53 alterations are associated with worse prognosis and may be relatively more resistant to chemotherapy and radiation (38). Additionally, accumulating lines of evidence suggest that breast cancer–associated gene 1 is involved in all phases of the cell cycle and regulates organized events during cell cycle progression (39). Our results favor a combination approach, activating both the promoter and enhancer regions for effective HLJ1 tumor suppressor–targeted therapy.

In conclusion, the transcription factors AP-1 and YY1 binding to the enhancer and promoter regions, respectively, activate and up-regulate the tumor suppressor, HLJ1. Elucidation of the roles and regulation mechanisms of tumor suppressors may facilitate the development of rational therapeutic targets for the suppression of cancer cell proliferation, angiogenesis, and metastasis.

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# Synergistic Activation of the Tumor Suppressor, HLJ1, by the Transcription Factors YY1 and Activator Protein 1

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